Annex II

Non obviousness of using an HIV-1 fusion inhibitor in scientific terms

As clearly exemplified below fusion peptide inhibitors based on the N-heptad repeat or the C-heptad repeat exhibit their potent inhibitory activity ONLY following pre-attachment of the virus to the target cell (i.e. following interaction between CD4 and co-receptors with gp120), and thus, only following conformational rearrangement of gp41. In other words, both viral and target cell membranes are in closc proximity to one another, and the distance between them is dictated by the size and conformational mobility of gp41.

Model of HIV Fusion Mechanism

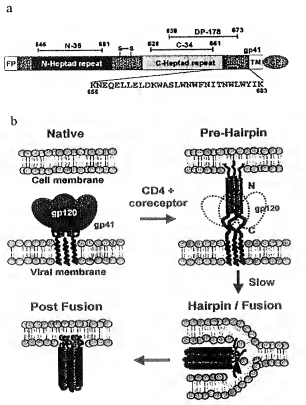

Figure 1. (a) A representation of the structure of gp41, the fusogenic subunit of HIV. CP, cytoplasmic domain; FP, fusion peptide; TM, transmembrane domain. The dimensions of the various domains are not drawn to scale. The approximate location of

the mAb 2F5 epitope is shown as a red line between the C-heptad repeat and the TM. (b) A representation of the current model of the HIV fusion process. The scheme is drawn according to literature consensus. (Weissenhorn, W. et al. (1997) Nature, 387, 426-430; Chan, D.C. et al. (1997) Cell, 89, 263-273); Exposure of the fusogenic subunit gp41 in the native state is believed to be minimal. However, the 2F5 epitope is exposed. The binding of gp120 to the target cell's CD4 and coreceptor triggers a conformational change within gp41 leading to the Pre-hairpin intermediate whieh frees the fusion peptides (i.e. N36 and C-34, T-20). The Pre-hairpin intermediate spans both membranes, with the gp41 TM domain anchored in the viral membrane, and the FP inserted into the target cell membrane. Inhibition by N (e.g. N36) and C-peptides (e.g. C-34, T-20) is thought to act at this level, by preventing transition to the hairpin/fusion structure (Eckert, D.M. & Kim, P.S. (2001) Annu. Rev. Biochem., 70, 777-810). More recently, T-20 was also shown to be capable of aeting at a post-lipid mixing stage (Kliger Y. et al. (2001) J. Biol. Chem., 276, 1391-1397). In this strueture, the C-heptad repeat folds back onto the N-heptad rcpeat to generate the six-helix bundles (trimer-of-hairpins) (Weissenhorn, W. et al. (1997) Nature, 387, 426-430; Chan, D.C. et al. (1997) Cell, 89, 263-273; Tan, K. et al. (1997) Proc. Natl. Aead. Sci. USA, 94, 12303-12308; Caffrey, M. et al. (1998) EMBO J., 17, 4572-4584). This structure is believed to correspond to the core of the fusion-active state of gp41 and shows similarity to the proposed fusogenic structures of envelopc fusion proteins from influenza (Bullough, P.A. et al. (1994) Nature, 371, 37-43), Moloney murine leukemia virus (Fass, D., and Kim, P.S. (1995) Curr. Biol., 5, 1377-1383; Fass, D. et al., (1996) Nat. Struet. Biol., 3, 465-469), simian parainfluenza virus 5 (Baker, K.A. et al. (1999) Mol. Cell., 3, 309-319), Ebola virus (Malashkevich, V.N. et al. (1999) Biochemistry, 96, 2662-2667), and simian immunodeficiency virus (Caffrey, M. et al. (1998) EMBO J., 17, 4572-4584; Yang, Z. et al. (1999) J. Struct. Biol. 126, 131-144; Malashkevich, V.N. et al. (1998) Biochemistry 95, 9134-9139).

Discussion:

(A) Steric Hindrance : Atomic Dimensions of Key Components

Albumin has an average molecular mass of 66,500, and a modified peptide:albumin bioconjugate will have a Mw of ~ 71 kDa. The tertiary structure of HSA alone derived from these X-ray measurements is a heart-shaped or equilateral triangular molecule 80 Å on a side, with average thickness of 30 Å, and a calculated molecular volume of about 88,249 $Å^3$ (He and Carter (1992) Nature (London) 358, 209-215). Conversely, the length of the central coiled-coil is approximately 115 Å (Weissenhorn, W. et al. (1997) Nature, 387, 426), and following conformational rearrangement of gp41, the N36/C34 complex (i.e. six-helix bundle or trimer of hairpins) formed during the Hairpin/fusion stage is composed of an overall dimension of ~35 Å in diameter and ~55 Å in height (Chan, D.C. et al. (1997) Cell, 89, 263-273). Furthermore, gp41 forms homo-oligomers thus further restricting the amount of solvent exposed surfaces within the N-heptad and C-heptad repeats. Taken together, it is highly unpredictable that an anti-fusion peptide attached

covalently to albumin would have essentially equivalent access to its target sequences within gp41 as the native peptide with a Mw of only 3-6 kDa (void of any covalent attachment to blood proteins).

(B) Comparison with Mw's of other known gp41-directed Fusion Inhibitors

The only other reported inhibitors which are directed towards the N-heptad or C-heptad repeats are T20) (37 amino-acid residues), DP-107 (38 aa's), N36 (36 aa's), T-649 (36 aa's), C14 (14 aa's), C34 (34 aa's), p38 (38 aa's), p26 (26 aa's), and siamycin I and II (21 aa's each). Therefore, these other known inhibitors have much lower Mw's than a bioconjugate of HSA with a modified antiviral antifusogenie peptide of the patent (~71 kDa). Again, it is highly unexpected that sueh a large bioeonjugate could have sufficient remaining activity (i.e. peptide's activity post-conjugation to albumin) and still be an effective inhibitor of HIV fusion.

(C) Mechanism of inhibition of DP178

Muñoz-Barroso, I. and co-workers (1998) J. Cell. Biol. 140, 315-323 describe that the mechanism of inhibition employed by DP178 is distinct to that used by DP107. More specifically, that DP178 possesses both a "low affinity" binding site on gp41 whieh prevents formation of the heterotrimeric coiled-coil structure, and a more recently discovered "high affinity" binding site on gp41 which occurs at a post-lipid mixing stage (i.e. prevents the transition from the lipid-mixing stage to the eytoplasm mixing stage). Using this model, these authors propose that DP178 bound the "high affinity" binding site does not prevent the close contact of the fusion peptides and COOH-terminal viral membrane anchors (i.e. both viral and host membranes are intermediately mixed). Due to its bulkier size, and thus poorer diffusion and translocation properties, relative to the free (native) peptide, a DP178:HSA bioconjugate would not be expeeted to have similar access to both the "low affinity" and "high affinity" binding sites as the free peptide. These results would, therefore, discourage Conjuchem from antieipating good activity towards inhibiting HIV fusion using DP178 attached covalently to serum albumin.

(D) Inability to Elicit Neutralization of HIV Infection using Monoclonal Antibodies

Inhibition of HIV fusion with the host cell has emerged as a viable therapeutic strategy, and rational design of vaccines interfering with this proeess is a prime target for antiviral research. HIV evolved a number of sophisticated immune-evasion strategies and, as a result, few broadly neutralizing antibodies active against primary isolates have been identified (Montefiori, D.C. (2001) Retroviral Immunology: Immune Response and Restoration; Pantaleo, G. & Walker, B.D., eds, pp. 191-211, Humana Press, Totowa, NJ; Trkola, A., et al. (1995) J. Virol., 69, 6609-6617). The human monoclonal antibody (mAb) 2F5 is the most thoroughly characterized. This antibody thus represents a

promising therapeutic agent, and the search for immunogens able to induce a 2F5-like immune response is a major goal of HIV vaccine programs. It neutralizes a broad array of primary HIV-1 strains (Trkola, A., et al. (1995) J. Virol., 69, 6609-6617; Conley, A.J., et al. (1994) Proc. Natl. Acad. Sci. USA, 91, 3348-3352; D'Souza, M.P., et al. (1997) J. Infect. Dis., 175, 1056-1062). The 2F5 MAb targets the fusogenic subunit of gp41 using residues 662-667, ELDKWAS, as its epitope (shown as small black line in Figure 1a) (Muster, T. et al. (1993) J. Virol., 67, 6642-6647; Purtscher, M. et al. (1996) AIDS, 10, 587-593; Zwick, M.B. et al. (2001) J. Virol. 75, 10892-10905). However, generation of a broadly neutralizing antibody response solely targeted to the 2F5 epitope is a relatively rare event during natural infection (Conley, A.J. et al. (1994) Proc. Natl. Acad. Sei. USA, 91, 3348-3352). Hence, it was suggested that the low level of exposure of this epitope, due to its location close to the viral membrane, may explain why generation of this antibody is a rare event in the course of natural infection despite the near-absolute sequence conservation (Conley A.J. et al. (1994) Proe. Natl. Acad. Sci. USA, 91, 3348-3352). Prior to 1999, therefore, it was the use of large gp41-directed protein inhibitors such as monoclonal antibodies, and not endogenous blood components such as serum albumin, which were expected to be efficient in blocking HIV fusion

However, a seeond gp41-directed monoclonal antibody discovered prior to 1999, designated NC-1, was generated using the model polypeptide N36(L6)C34 which folds into a stable six-helix bundle (Jiang, S. et al. (1998) J. Virol., 72, 10213-10217). NC-1 was found to bind specifically to both the α-helical core domain (i.e. six-helix bundles, trimer of hairpins) and the oligomeric forms of gp41 in a conformation-specific manner. Despite this, however, NC-1 had no detectable inhibitory activity for blocking fusion between HIV-1 IIIb-infected cells and uninfected MT-2 cells, even at a concentration of 200 μg/ml. From this, it was postulated that the NC-1 epitopes may be buried in the gp120 and gp41 complex and therefore not accessible to the antibody following viral attachment to the uninfected host cells (Jiang, S. et al. (1998) J. Virol., 72, 10213-10217).

Hence, the failure of the NC-1 monoclonal antibody to control the infection for reasons of lack of accessibility again deters the skilled person from making a large fusion inhibitor since it would not be expected to have sufficient access to be able to prevent viral fusion.

(E) Active Participation of Albumin in the Inhibition of Fusion

According to D4, it has been demonstrated that an important loss of activity was measured upon binding of the dynorphin albumin conjugates to the various opioid receptors. The loss of activity could be measured in terms of loss of in vitro binding activity. Despite the loss, the conjugates were still sufficiently active to provide analgesia in animal models of mice and dogs.

The loss of activity observed with the dynorphin derivatives in D4 is typical of the technology when applied to G protein-coupled receptors (GPCRs). Other GPCR target

peptides have produced similar potency reduction while retaining sufficient clinical activity to maintain commercial interest (up to 100-fold loss of potency with DAC:GLP-1 drug candidate now in human clinical Phase 2).

In the case of the HIV fusion peptides described in the application, we were surprised to observe that, in several instances, no loss of activity due to the bioconjugation process was observed. Further, in one instance the peptide conjugate appears to be more potent in a fusion assay than the free parent peptide. This highly unexpected result in view of D4, D5 and D6 provide the invention with a decisive advantage.

Conclusions

In the year 1999, the molecular mechanisms of HIV fusion were known to have a strict requirement that both viral and host cell membranes need to be in close proximity to one another so as to expose the N-heptad and C-heptad repeats. It would not have been expected that exogenous peptide:albumin bioconjugates would be successful in blocking conformational rearrangements of gp41, particularly in light of the fact that gp41 is oligomeric and its conformations transitions are intramolecular processes. Furthermore, that such large bioconjugates are found to be essentially equipotent to the native peptide as well as to other known fusion inhibitors having much smaller molecular weights. An improved understanding in the anti-fusion mechanisms of T-20 have only been obtained recently (i.e. following the year 1999; (Muñoz-Barroso, I. et al. (1998) J. Cell. Biol. 140, 315-323 which make the data with T-20:HSA conjugates even more remarkable. Finally, the recent failures in eliciting gp41-directed antibody neutralization exemplifies the difficulty in designing very large protein-based inhibitors that are targeted to the gp41 structure.